Exposure of *Desulfovibrio vulgaris* Cells to Chromium(VI) Temporarily Decouples Lactate Oxidation from Sulfate Reduction

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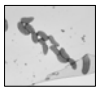


Abstract

Desulfovibrio vulgaris is an anaerobic sulfate-reducing bacterium (SRB) able to reduce toxic heavy metals such as chromium and uranium, and *D. vulgaris* represents a useful SRB model for the bioremediation of heavy metal contamination in anaerobic sediments. Although much work has focused on Cr and U reduction via individual enzymes (e.g., hydrogenase, cytochromes), less is known about the cellular response to heavy metals in *Desulfovibrio* species. When exponential-phase cells were washed to remove hydrogen sulfide carry-over and inoculated into fresh medium with different levels of Cr(VI), lag time increased as the levels of Cr increased. Cells lagged approximately 5, 40, and 55 h in the presence of 20, 50, and 100 µM Cr, respectively. When cells were transferred to 50 µM Cr, Cr(VI) levels declined within 2 h and lactate was consumed, but sulfate did not decline until growth was initiated approximately 40 h later. Lactate continued to be consumed at a slow rate during the lag but sulfate levels remained unchanged. When cell growth was initiated, lactate utilization rate increased, sulfate was consumed, and acetate levels increased. Similar trends were observed for cultures treated with 20 or 100 µM Cr(VI) that corresponded to the different lag times. During the lag phase when lactate was consumed, the production of hydrogen was detected. However, the amount of hydrogen produced with or without Cr(VI) was not significantly different. These results indicated that hydrogen production alone could not account for the utilization of lactate in the absence of sulfate reduction. The results indicated that lactate oxidation was decoupled from sulfate reduction in the presence of Cr(VI). In addition, the Cr(VI) exposure caused a recovery time in the cells even after the Cr(VI) was reduced, and cells had prolonged recovery times when the initial Cr(VI) levels were increased.

Introduction

A number of anthropogenic activities have caused extensive Cr contamination in both soils and water. Cr is the third most common pollutant at hazardous waste sites and the second most common inorganic contaminant after Pb. Cr(VI) is water-soluble, mutagenic, and carcinogenic, but Cr(III) is less-soluble, less-toxic, and less mobile. A variety of studies (6, 14, 16, 34, 39) have documented the ability of SRB, including *Desulfovibrio* spp., to reduce toxic metals such as U(VI) and Cr(VI) enzymatically, a process that results in the production of less water-soluble species. The modification of solubility properties caused by changing the redox state of the metal presents itself as a potential avenue for bioremediation of contaminated groundwater and soils. Previous research specifically points toward SRB as environmentally-relevant experimental systems for the study of heavy metal and radionuclide reduction (2, 3, 33). Sulfate-reducers provide several advantages with respect to heavy-metal reduction including the presence of sulfate in a variety of environments and the protection of immobilized heavy metals from oxidation with iron sulfides (mackinawite).

Previous work has shown that *D. vulgaris* requires hydrogen sulfide, hydrogenases and cytochrome *c3* for the reduction of Cr(VI) (Lovely et al., 1994; Chardin et al., 2002), and that Cr(III) can be detected on the cell surface as well as between the cytoplasmic and outer membranes (Goulhen et al., 2005). Microcalorimetry was used to observe energy production without growth in the presence of Cr(VI) (Chardin et al., 2002), but acetate and sulfate levels were not reported. In addition, the re-establishment of growth was not monitored, and the growth medium was not defined. In comparison, recent work has shown that U(VI) inhibited sulfate-loss, and that both Fe(III) and U(VI) inhibited lactate-mediated sulfate reduction (Elias et al., 2004). However, it is not known if a lag time ensues with respect to cell growth post-treatment with U(VI) or Fe(III).

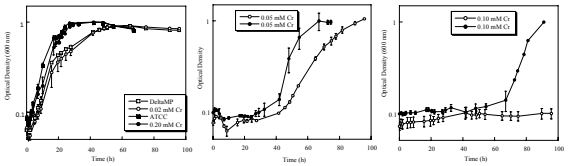
Materials and Methods

Bacterial strains and growth conditions. Cultures of *D. vulgaris* ATCC 29579 were obtained from Terry Hazen (Lawrence Berkeley National Laboratory). *D. vulgaris* ATCC 29579 was grown in a batch tubes or bottles sealed with butyl stoppers in LS4D medium. LS4D is a defined medium with approximately 50 mM Na2SO4, 60 mM NaC3H6O3, 8.0 mM MgCl2, 20 mM NH4Cl, 2.2 mM KHPO4, 0.6 mM CaCl2, Thauers vitamins, trace minerals, 30 mM PIPES buffer, 0.06 µM resazurin, and 10 mM NaOH to pH to 7.2. Triplicate columns were inoculated with a 10% (v/v) inoculum from the same culture of *D. vulgaris* to similar initial optical densities (600 nm). Cultures were incubated in a 30°C water bath and samples were collected aseptically at various time points via a septum. Protein was analyzed with the Lowry method and bovine serum albumin (Pierce Biochemicals) as a standard. Carbohydrate was determined with a cysteine-sulfuric acid colorimetric assay as previously described (9). Lactate, acetate, and sulfate concentrations were measured via ion chromatography (Metrohm-Peak) with a Metrosep organic acid column and a Metrosep Anion Supp 5 column, respectively. All assays were done in duplicate and the variation was less than 10%.

ATCC or ΔMP pre-culture cells were grown in anaerobic tubes with LS4D culture medium under N2. The growth was continued until the pre-cultures reached the OD of 600 nm of 0.5-0.7 or 0.9 (35 hrs of culture for ATCC, and longer for ΔMP) for exponential and stationary cultures, respectively. Cells were harvested by centrifugation at RT, for 10 min in under anaerobic conditions. The pellets were washed twice, suspended in fresh LS4D medium and used immediately for inoculation. Measures were taken to avoid oxygen exposure. Washed cells were inoculated into 0, 0.02, 0.05, 0.1 mM Cr(VI). Cr(VI) was added to the cultures and first samples corresponding to T0 were immediately withdrawn and analyzed. Cultures were incubated at 30°C, samples were recovered with a N2 flushed syringe (injecting the same volume of N2 as the volume of the sample) every hour for first 3 hours, and then every 5 hours, approximately.

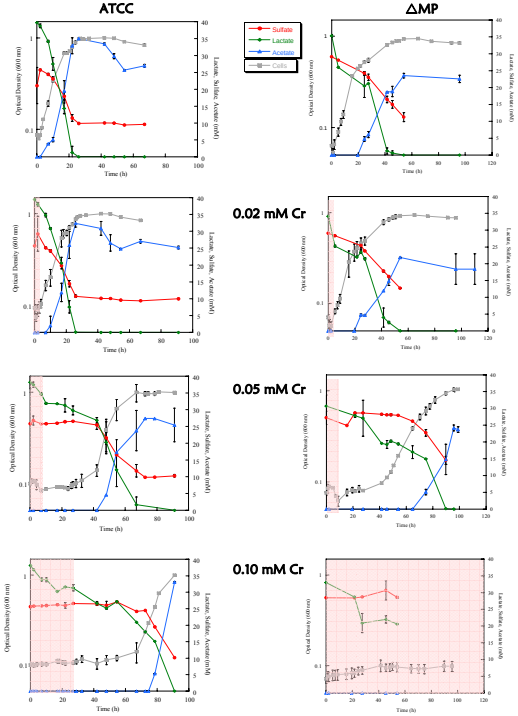
The Cr(VI) levels were determined as previously described (Viamajala et al., 2002). Samples were used immediately for determination of Cr(VI) levels. All samples were always treated immediately after being withdrawn from cultures. The assay was performed by adding 0.1 ml of sample to 0.9 ml of Chroma Ver3 solution. The mix was then incubated for 15 min at RT, and OD was measured at 540 nm. Cr(VI) calibration was obtained in the range 0-0.1 mM Cr(VI).

Cellular Response to Cr(VI) Differs Between Wild-Type and ΔMP



When cells were harvested from exponential growth, anoxically washed, and re-inoculated into fresh medium with increasing levels of Cr(VI), cell growth lagged in the presence of 0.05 and 0.10 mM Cr. Little effect was observed for 0.02 mM Cr. Cells displayed an approximately 24 h and 60 h lag with 0.05 mM and 0.10 mM Cr, respectively. Once growth was initiated, the growth rate and final yields were similar to the culture without Cr addition. In addition, the strain without the megaplasmid (ΔMP), was more susceptible to 0.05 mM Cr(VI), and could not tolerate 0.10 mM Cr(VI).

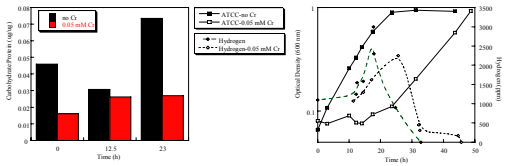
Cellular Response to Cr(VI) Differs Between Wild-Type and ΔMP



Cells lagged approximately 5, 40, and 55 h in the presence of 20, 50, and 100 µM Cr, respectively. When cells were transferred to 50 µM Cr, Cr(VI) levels declined within 2 h and lactate was consumed, but sulfate did not decline until growth was initiated approximately 30 to 40 h later. Lactate continued to be consumed at a slow rate during the lag, but sulfate levels remained unchanged and growth was not observed. When cell growth was initiated, lactate utilization rate increased, sulfate was consumed, and acetate levels increased. At a Cr(VI) level of 100 µM, the cells consumed approximately 10 mM lactate, but sulfate was not reduced and growth was not observed. After nearly 60 h, growth resumed with a concomitant production of acetate. These results indicated that lactate oxidation was decoupled from sulfate reduction and growth, and that reducing power was utilized for non-growth expenditures. In addition, the results suggested that the reduction of Cr(VI) caused more than a temporary re-direction of electrons from sulfate, and that cellular effects continued post-Cr(VI) reduction.

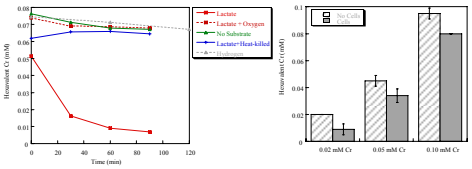
The ΔMP strain could obtain similar final yields as wild-type, but the growth rate was slightly slower with a bimodal pattern. As with wild-type, 20 µM Cr had little effect on ΔMP cells. At 50 µM Cr, Cr was reduced within 2 h, and growth lagged for approximately 40 h. However, the acetate production lagged for a longer time period compared to wild-type cells. At 100 µM, ΔMP cells did not recover, and growth was never observed. These results indicated the ΔMP cells were more susceptible to Cr(VI) exposure.

Carbohydrate to Protein Ratio and Hydrogen Production



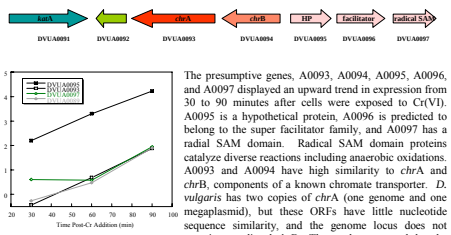
Microorganisms within the *Desulfovibrio* genus have been observed to produce glycogen, and glycogen production might explain the utilization of lactate without growth. However, the carbohydrate to protein ratio did not increase for cells exposed to 50 µM Cr(VI), and a significant amount of extracellular carbohydrate was not detected (data not shown). In addition, Cr(VI) treated cells did not accumulate increased levels of hydrogen compared to non-treated cells. These results indicated that glycogen and hydrogen production could not explain the oxidation of lactate without growth or sulfate reduction.

Whole-Cell Cr(VI) Reduction



Whole-cells could reduce Cr(VI) in the presence of lactate but not hydrogen during the time of assay. Cells, alive and heat-killed, did bind small amounts of Cr(VI), but heat-killed cells did not reduce significant amounts of Cr(VI). The results suggested that Cr(VI) reduction mechanism does not involve hydrogenases as *D. vulgaris* cells reduced Cr(VI) efficiently in absence of hydrogen and hydrogen addition has no significant effect Cr(VI) reduction.

Up-Expression of Megaplasmid ORFs in Response to Cr(VI)



The presumptive genes, A0093, A0094, A0095, A0096, and A0097 displayed an upward trend in expression from 30 to 90 minutes after cells were exposed to Cr(VI). A0095 is a hypothetical protein, A0096 is predicted to belong to the super facilitator family, and A0097 has a radial SAM domain. Radical SAM domain proteins catalyze diverse reactions including anaerobic oxidations. A0093 and A0094 have high similarity to *chr*A and *chr*B, components of a known chromate transporter. *D. vulgaris* has two copies of *chr*A (one genome and one megaplasmid), but these ORFs have little nucleotide sequence similarity, and the genome locus does not contain a predicted *chr*B. The results suggested that the above megaplasmid genes may play a role in chromate resistance, and could be significant after the initial Cr(VI) reduction to Cr(III).

Major Conclusions:

- Cells were more susceptible to the effects of Cr(VI) when washed before inoculation (i.e., H_2S removal)
- Cell growth did not lag in the presence of 20 µM Cr(VI), however, lag times increased with 50 (40 h) and 100 (55 h) µM Cr(VI)
- A strain (△MP) without the megaplasmid displayed increased susceptibility to Cr(VI), and did not survive exposure to 100 µM Cr
- Preliminary transcriptomic data suggested that five ORFs on the megaplasmid were up-expressed upon Cr(VI) exposure, and included a presumptive hypothetical protein, a putative super facilitator family, a presumptive radial SAM domain, and putative *chr*A and *chr*B.
- During the growth lag, lactate was still consumed, but sulfate was not reduced and acetate was not produced
- Data indicated that lactate oxidation was decoupled from sulfate reduction; however, hydrogen levels or carbohydrate levels could not explain the lactate utilization without growth
- In addition to the decoupling of lactate oxidation and sulfate reduction, Cr(VI) exposure caused a recovery time in the cells long after the reduction of Cr(VI)
- Results suggested that cells required time to deal with the oxidizing stress of the Cr(VI), and/or deal with the production of Cr(III)
- Current work includes the determination of redox conditions during Cr(VI) exposure and the cellular mechanisms of reduction and transport

Acknowledgements

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